THE OCCURRENCE OF TOXIGENIC ANAEROBES, ESPECIALLY *CLOSTRIDIUM BOTULINUM*, IN SOME ENGLISH SOILS

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Few examinations of British soils for the presence of *Clostridium botulinum* seem to have been made. Meyer & Dubovsky (1922*b*) obtained 8% certainly positive, with a possible 13% positive results, out of sixty-four samples of English soil shipped to America and examined there, all positive samples containing *Cl. botulinum* type B. Leighton & Buxton (1928) tested 100 samples of soil from all the counties of Scotland, obtaining 4% positive samples, two containing type A, one type B, and one neutralized by both type A and type B anti-toxin. Three of the positive samples came from pastureland, one from ploughed land.

In the present experiments samples of soil from 106 localities have been examined for the presence of *Cl. botulinum*, and incidental information on the distribution of some other anaerobes obtained concurrently. The samples have mostly been taken from the south-easterly part of England.

Technique

Quantities from about 25 to 100 g. of the soil, collected usually in about ½ kg. lots in sterilized tins, were weighed into stout glass bottles. Sufficient saline, generally 25–100 c.c., was added to moisten the sample thoroughly, clay soils being broken up with a glass rod, the whole vigorously shaken to yield a thick suspension, and a layer of minced beef (50–100 g.), prepared as for Robertson's medium, added. The bottles were then stood in water at room temperature, brought as quickly as possible to 80° C., and maintained there for periods of ½–1 hr. 50–100 c.c. of suitable medium, generally Veillon broth (beef peptic digest liver broth, *p*H 7·4, containing 0·2% dextrose), were then added, the bottles cooled, closed with bungs, evacuated at the oil pump, and sealed with screw-clips, joints being waxed. Incubation was for periods of 6–11 days at 25 or 37° C. Gas production was often sufficiently vigorous to blow the bungs of the bottles in 3–4 days, after which the bottles were plugged with cotton-wool until ready for examination.

For testing the enrichment culture thus obtained, 20 c.c. of the fluid were centrifuged at 5000 rev./min. for 15 min., and quantities of 0·1–0·5 c.c. injected intraperitoneally into mice. All cultures lethal within a period of 10 days were re-examined, inoculations being made in the case of suspected cultures

into mice previously immunized with polyvalent (A + B) *Botulinum* anti-toxin, or else inoculum and anti-toxin were administered together as convenient. In addition, *Tetanus* anti-toxin and polyvalent 'gas-gangrene' anti-toxin (against *Cl. welchii*, *Cl. septique* and *Cl. oedematiens*) were used when necessary, and separate *Botulinum* A and B anti-toxins used for final typing. The method has been checked in certain cases by feeding positive cultures to mice, and from one '*Botulinum* A positive' sample the organism was isolated in pure culture. In certain cases centrifugates have been Seitz filtered, but this was not in general necessary, and when used led to a serious decrease in toxicity.

The chief drawback to the method is the liability of some of the mice to die within short periods (5–30 min.) after inoculation, with characteristic symptoms, namely, marked distress in breathing, convulsive spasms of the limbs and whole body, the body being sometimes flung off the bench, respiration becoming slow, the mouth opening with each inspiration, the animal turning on its side and extending all its limbs in a final spasm, eyes often becoming opaque 'porcelain white'. A similar effect following intraperitoneal inoculation was noted by Dubovsky & Meyer (1922*a*), but we have found that the same result also follows subcutaneous inoculation in the back of the animal. We assumed that a toxic amine is responsible for these deaths. The post-mortem findings suggested that the toxic substance was probably not histamine. The lungs were not distended. There was no lysis in blood from the heart. Boiling vigorously for 10 min. had little or no effect on the toxicity of such centrifugates, nor were they neutralized by any of the anti-sera mentioned above. There was generally a sharp end-point to the toxic effect on decreasing the inoculum, e.g. 0·4 c.c. lethal within 30 min., 0·3 c.c. no effect. On the other hand, dilutions of *Botulinum*-toxic cultures over a much wider range merely delay the onset of characteristic symptoms. While this phenomenon has meant that certain cultures have had to be abandoned, there can be no confusion between it and the action of a 'true' toxin, especially *Botulinum* toxin. The latter was consistently destroyed by boiling for 10 min., was generally lethal in small amounts, and the injection of say 1000 M.L.D. did not produce characteristic symptoms until 3–6 hr. after inoculation. The more obvious of these symptoms are distressed breathing, a typical falling in of the flanks until a marked 'wasp-waist' is seen, paralysis of one or more limbs, and finally, death.

Inevitably a certain number of cultures have failed to give entirely satisfactory evidence in the typing with A and B anti-toxins and the necessary controls. The most common were weakly toxic cultures satisfying the preliminary test with polyvalent anti-toxin, but failing, after an interval, to give consistent results with separate A and B anti-sera, and cultures containing *Cl. tetani* or other organisms as well as *Cl. botulinum*. Such cultures have been classed as possibly positive.

RESULTS

The results are summarized in Table 1. It may be seen that of samples of soil from 106 localities, 37% were shown to contain toxigenic anaerobes, *Cl. botulinum* being certainly found in 5% of the samples and possibly occurring in 14% of the samples, *Cl. tetani* being found in 10%, and organisms of the 'gas-gangrene' group in 8%. Of the cultures, 8% were toxic from unknown cause, but post-mortem examination suggested that a number of the 'unknowns' were probably attributable to *Cl. welchii*. Of the five definitely positive *Botulinum* samples four were shown to contain type A, one type B.

Table 1

County	Type of soil	No. of localities examined		No. of toxic samples		*Botulinum*	*Tetanus*	'Gas gangrene'	Unknown
Essex	Woodland	4	6	0		—	—	—	—
	Arable	2		—		—	—	—	—
Bedford	Arable	10	11	2		?2	—	—	—
	Grass	1		—		—	—	—	—
Berkshire	Arable	4		1		—	—	—	1
Buckingham	Arable	3		3		?1	1	—	1
Cambridge	Garden	15	43	3	16	?1	2	—	—
	Wicken Fen	6		0		—	—	—	—
	Arable fen	12		10		?4	1	5	1
	Arable (clay or chalk)	10		3		1A	1	—	1
Hampshire	Arable	4	5	3	4	1A	1	—	1
	Grass	1		1		1A, 1B	—	—	—
Kent	Garden	8		1		—	—	—	1
Leicester	Arable	2		0		—	—	—	—
Lincoln	Arable	1		0		—	—	—	—
Norfolk	Arable	2	7	1	4	—	—	1	—
	Garden	1		1		—	1	1	—
	Grass	4		2		1A	—	—	1
Northampton	Grass	2	4	1	1	?1	—	—	—
	Arable	2		0		—	—	—	—
Nottingham	Arable	1		0		—	—	—	—
Oxford	Arable	2		1		—	—	—	1
Rutland	Arable	1		0		—	—	—	—
Warwick	Garden	2	5	2	3	—	1	1	—
	Arable	3		1		—	1	—	—
Wiltshire	Arable	3		1		—	1	—	—
		106		37		14	10	8	8

Note. The fact that the total of the last four columns is greater than the total of the preceding column is due to some samples containing more than one toxigenic organism.

Type A was obtained from grassland at Alton, Hampshire, from common land at Ringland, Norfolk, from a cornfield near Sutton Scotney, Hampshire, and from freshly ploughed grass near Ely, Cambridgeshire. Type B was obtained from another lot of the sample of grassland at Alton, Hampshire, which also yielded type A. It may be noted that of the five certainly positive cultures three were from grassland, and one from freshly ploughed grass.

The cultural and morphological characteristics of the type A strain isolated from grassland, Alton, Hampshire, and twice replated were as follows.

Rod about $4 \times 1\,\mu$, subterminal spores considerably swelling the rod, spores not quite circular, about $1{\cdot}5 \times 1{\cdot}8\,\mu$, longest axis parallel with the axis of the

rod. Spores are freely formed in Robertson's medium in 3–4 days. There are few or no spores in 24 hr. at 37° C. The rods are motile in 24 hr. culture in Robertson's medium, the non-sporing rod having parallel sides and rounded ends. The rods become somewhat cigar-shaped as sporulation commences. They are Gram-positive in young culture. The organism grows easily in deep stabs, and in shake culture to within about 2 cm. of the surface. The agar is disrupted and blown to the plug. Deep colonies are disk-shaped with an entire edge and a somewhat darker centre. Often the disks appear to start growth in two or three planes together, giving the colony a somewhat star-shaped appearance in section. Acid and gas is produced in nutrient broth containing dextrose or maltose, the *p*H being lowered from 7·4 to between 4 and 5, and salicin is also attacked, the *p*H of a culture grown anaerobically (1% sugar) changing from 7·4 to about 5 in 24 hr. Dextrin too is broken down, the *p*H change in this case being from 7·4 to about 6. Sucrose, lactose, galactose, mannitol, xylose, arabinose, dulcitol and raffinose are not attacked. Marked turbidity is produced in Robertson's medium and in broth in 24 hr., after which the medium tends to clear with a sediment at the bottom. Digestion of meat in Robertson's medium, if it occurs at all, is slight, and some blackening may be apparent in old cultures (one week or more). Coagulated egg-white in broth is little, if at all, digested, clotted serum is digested, and gelatin is liquefied. The minimum lethal dose for a mouse of about 25 g. by intraperitoneal injection from a 24 hr. Robertson's culture at 37° C. is 0·0001 c.c.

Comment

Essentially, the technique used is that of Meyer and his colleagues (see Dubovsky & Meyer, 1922*a*). It would be desirable to examine variations of the method for English conditions, since it is known that various strains of *Cl. botulinum* differ in the heat resistance of their spores, their optimal temperatures for growth, and that in the presence of other organisms toxic cultures may not always be obtained. Circumstances, however, did not allow a systematic examination of these points, but the following data were obtained. 130 cultures heated at 80° C. for 1 hr. and incubated at 25° C. (Veillon broth) yielded 18% toxic cultures (1% possible *Botulinum*), 79 heated at 80° C. for ½ hr. and incubated at 35° C. (Veillon broth) gave 29% toxic cultures (6% possible *Botulinum*), and 47 heated at 80° C. for ½ hr. and incubated at 35° C. (nutrient broth) gave 42% toxic cultures (2% possible *Botulinum*). It was also demonstrated that the addition of spores of a stock culture of *Cl. botulinum* type A to soil cultured by the above methods did in fact yield a toxic culture, but the minimum number of spores that would give a positive result was not determined.

It may be seen that the results obtained are more in agreement with those of Leighton & Buxton obtained in 1928 for Scottish soils in suggesting that type A is commoner than type B, than with the view of Meyer & Dubovsky (1922*b*) that type A is uncommon, in European soils. On the other hand, the

fact that four out of five positive cultures came from grassland (if one recently ploughed grass is included), as did three out of four of Leighton & Buxton's, agrees with Meyer & Dubovsky's finding that *Cl. botulinum* is less easily isolated from cultivated soil than from pastureland. It would seem, however, that before the latter authors' view that cultivation suppresses *Cl. botulinum* is accepted, it must be shown that the smaller number of *Botulinum*-positive cultures from cultivated soils is not due to the fact that the presence of so many other organisms either inhibits the growth of, or destroys the toxin of, any *Cl. botulinum* that may be present. Thus, it is interesting to note that no culture from six samples of soil from Wicken Fen was toxic, but cultivated Fenland soils yielded a high proportion (10/12) of toxic samples, five being definitely positive for organisms of the 'gas-gangrene' group, one definitely positive for *Cl. tetani*, and four doubtfully positive for *Cl. botulinum* (mice dying with characteristic symptoms from fresh culture, but subsequent examination failing to give clear-cut results with anti-sera).

SUMMARY

1. Examination of samples of soil from 106 localities by enrichment culture technique gave 37 % toxic samples, the percentage containing *Clostridium botulinum* ranging from a certain 5 % to a possible 14 %.

2. *Cl. botulinum* type A was shown to be present in four of the five positive samples, type B in one. Characteristics of a pure culture of type A are given.

3. Incidental data on the occurrence of some other toxigenic anaerobes are given.

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